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(54) Title: STABLE COLLAGENASE COMPOSITIONS AND METHODS FOR THEIR PREPARATION

(57) Abstract

Stabilized collagenase compositions and methods for their preparation are disclosed. A collagenase solution is mixed with a buffer and a stabilizer and lyophilized. The stabilizer can be a protein or oligosaccharide. Stabilized collagenase compositions retain a high degree of hydrolytic activity and solubility following lyophilization and long term storage.

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STABLE COLLAGENASE COMPOSITIONS AND METHODS FOR THEIR PREPARATION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to stabilized enzyme compositions and methods for preparing and storing stabilized dry enzyme compositions. More particularly, the present invention is directed toward the proteolytic enzyme, collagenase, and methods for stabilizing collagenase during lyophilization procedures and for producing dry collagenase compositions which are suitable for long-term storage.

2. Description of Relevant Art

Proteolytic enzymes are widely utilized in a variety of laboratory and clinical applications. Typically these applications involve cell dissociation and related therapeutic procedures which are benefitted by the ability of proteolytic enzymes to hydrolytically break up or loosen connective tissue networks. For example, bacterial collagenase derived from Clostridium histolyticum has been used to disperse cells in laboratory tissue culture applications. Additionally, collagenase has demonstrated utility in cell isolation procedures such as those associated with isolating pancreatic islets and dispersing a variety of tumor cells. Collagenase has also found use in clinical applications, for example, topically for the treatment of burns, ulcers and other wounds. Collagenase is also utilized in the treatment of Peyronie's disease and as an adjunct to cryoprostatectomy for the removal of retained cryoslough, intervertebral discolysis, and in ophthalmic surgery.

Most commercial collagenase is derived from the bacteria Clostridium histolyticum and, in its crude form, differs widely in hydrolytic activity and purity. Unc ntrolled amounts of impurities found in crude

collagenase include contaminating bacterial materials, pigment, toxins, and proteolytic enzymes, including clostripain, trypsin, and caseinase. A number of problems are associated with using crude collagenase to digest tissue and isolate embedded cells. For example, proteolytic enzyme impurities will cause the catalytic degradation of the collagenase composition. Moreover, toxins associated with crude collagenase can be a serious problem for procedures involving both in vivo and in vitro applications. Toxins can disrupt cell membranes, destroy cell viability and generally lower cell yield. Bacterial materials may include variable amounts of bacterial DNA, which potentially can cause immunologic and tumorigenic problems when isolated cells or tissue digestion procedures involve in vivo applications. Finally, noncollagen-specific protein impurities found in crude collagenase may act as sensitizing antigens, which can cause anaphylactic shock if administered to patients.

In order to overcome problems associated with the use of commercial sources of crude collagenase, procedures for efficiently and effectively isolating and purifying collagenase have been developed. Most collagenase purification procedures involve chromatographic separation of collagenase from contaminating proteolytic enzymes, toxins, and various unreactive components of crude collagenase. Typically, following purification, the collagenase is lyophilized to provide a dry composition for storage. Even though purified and partially purified collagenase is more stable in its dry form than in solution, the lyophilization procedure and subsequent dry storage is known to result in degradation of the collagenase. This degradation is exemplified by a substantial decrease in its solubility in aqueous systems and a decrease in its specific activity.

Methods for stabilizing proteins and cell preparations using a variety of stabilizers and/or buffer matrices have been reported. For example, U.S. Patent No.

4,315,002 discloses the use of d xtrose to stabilize interleukin-2 and U.S. Patent No. 4,962,091 reports the use of human serum albumin and dextrose as stabilizers for interferon. Yet neither disclosure specifically contemplates the stabilization of proteolytic enzymes including purified collagenase. As dextrose and human serum albumin have diverse stabilization capabilities depending on the properties of the protein to be lyophilized, there is no indication that these reported measures would be effective in maintaining collagenase activity.

Extended stabilization of collagenase was reported in U.S. Patent No. 4,732,758 through the use of hydrolysates of collagen being combined with the enzyme prior to freeze-drying. However, unlike the present invention, this method involved the direct use of an altered substrate which could be expected to interact and protect the collagenase during desiccation. In the present invention there was no indication that oligosaccharides or non-substrate proteins confer any beneficial properties if added prior to lyophilization.

Other authors have reported the attempted stabilization of phospholipid bilayers and proteins through the use of sugars (Crow et. al., Cryobiology 24, 455-464 [1987]) including disaccharides. Though stabilization of phosphofructokinase was achieved, the authors indicate that different enzymes will react differently and various sugars give substantially dissimilar results. Yet, even if the initial post-lyophilization activity of a protein in the presence of a suspected stabilizer is high, there is often a substantial reduction in activity after long term storage. With respect to collagenase, there is no reported stabilizer or combination of stabilizers shown to prevent the degradation of purified or partially purified collagenase during lyophilization and dry storage.

Accordingly, it is an object of the present invention to provide collagenase compositions having improved stability.

It is another object of the present invention to provide methods for the maintenance of collagenase activity during the lyophilization process and long term storage.

It is an additional object of the present invention to provide stabilized collagenase compositions which retain their hydrolytic activity and aqueous solubility following lyophilization and long-term storage.

SUMMARY OF THE INVENTION

The present invention accomplishes the above objectives by providing stabilized collagenase compositions and methods for their preparation. Stable collagenase compositions, prepared according to the methods of the present invention, retain a high degree of hydrolytic activity and solubility following lyophilization and long term storage. Because these compositions maintain their hydrolytic activity following lyophilization and storage, they are useful in tissue digestion and cell isolation applications which require predictable enzyme performance.

More particularly, the present invention provides for incorporating selected proteins or oligosaccharides with collagenase to stabilize the collagenase. Through the addition of selected concentrations of these components under favorable conditions, the collagenase retains a substantially higher specific activity than it otherwise would have after lyophilization. Further, this composition will continue to retard the degradation of collagenase, ensuring that a higher level of enzyme activity is maintained over extended periods of time. In addition, the use of these compositions prevents denaturing during lyophilization and storage, thus incr asing the overall solubility of the desiccated

enzyme. The consistent specific activity and retention of physical parameters is critical to the development of laboratory reagents and therapeutic clinical applications involving collagenase.

As the present invention will enhance the stability of collagenase irrespective of any contaminants, there is no minimum required level of purification. However, a reduction of contaminants will result in a corresponding decrease in the rate of degradation and an increase in the level of stability achieved. Preferably, any collagenase preparations suitable for use in the compositions of the present invention will be substantially free of pigment, bacterial materials, toxins, and noncollagen-specific enzyme activity. Therefore, a preferred embodiment of the present invention involves the use of collagenase that has been purified to some extent. This may be accomplished in any one of a number of ways well known in the art such as fractionation, centrifugation, and chromatography, or a combination thereof.

Following any desired purification of the collagenase, the enzyme is mixed with the stabilizer or stabilizers in a suitable aqueous buffer solution, such as a buffered electrolyte solution, which may contain a variety of different salts to facilitate enzyme solubility, and if desired, a surfactant. Commonly, the mixture is adjusted to the desired pH using a buffer, such as a phosphate or Tris buffer, tris(hydroxymethyl)aminomethane, for example. If a surfactant is desired, a nonionic surfactant preferably is employed, usually at concentrations of about 0.01% to about 0.05% by weight.

While the stabilization of individual proteins in desiccated samples has long been reported, the mechanisms involved have yet to be elucidated. It has been suggested that the stabilization of proteins in solution may be a result of the exclusion of structure-d stabilizing compounds from contact with the protein surface. In this

model, such contact w uld increase the chemical potential of the protein solution by denaturing the protein, which would result in an increase in the thermodynamic potential of the system. However, this explanation does not readily extend to desiccated compositions. Other studies have indicated that the subunit orientation of the stabilizing sugar and pre-lyophilized cation concentration of the solution is important in stabilizing protein samples. No proposed mechanism has been generally accepted by the scientific community at this time and no realistic predictions can be made either with regard to the feasibility of stabilizing selected proteins, or what compounds may be useful in this respect.

According to the present invention, collagenase may be stabilized by the addition of certain proteins such as globulins or certain oligosaccharides, such as maltose, trehalose, and sucrose. Although globulins such as human serum albumin and bovine serum albumin are preferred, other animal proteins can be used as well. With respect to the sugars, in general, any non-reducing sugar can be used in addition to the preferred oligosaccharides. The protein or sugar stabilizers can be present singly or in combination. In particular, the present invention combines one or more stabilizers with collagenase in an aqueous buffer for the purpose of stabilization.

While the concentration of stabilizer can vary, typical values are about 1 mg/mL to about 100 mg/mL of the stabilized collagenase solution. Preferably the mixture of stabilizer and buffer is filtered to remove possible contaminants before combining with the collagenase. However, it is possible to obtain satisfactory results by adding the stabilizer in solid form after the collagenase has been mixed into a predetermined volume of aqueous buffer. For the purposes of this application the volume of liquid used to mix the stabilizer with the collagenase in the final step of the process before lyophilization is referred to as the final mixing volume. The aqueous

buffer, containing the mixture of stabilizer and collagenase is gently mixed to insure homogeneity of the solubilized components.

Once a homogeneous solution is obtained, the liquid is aliquoted and lyophilized. Lyophilization employs a vacuum to remove any water present in the frozen aqueous solution and results in the deposition of a dry, concentrated film or powder. In this case the concentrated powder consists primarily of the collagenase and stabilizer. In addition, the salts used to buffer the solution and small amounts of impurities may also be present. After initial freezing of samples, the lyophilization process may be carried out over a range of temperatures extending from 4°C to 37°C and under a high degree of vacuum, but for the present invention is preferably performed at room temperature (25°C). The resulting powder, with stabilized enzymatic activity, may then be packaged in a variety of forms depending on the intended use.

Further objects, features, and advantages of the present invention will become apparent to those skilled in the art from a consideration of the following detailed description of exemplary embodiments.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

The present invention provides improved stabilization of collagenase and a corresponding increase in specific activity during lyophilization and long-term storage when compared with non-stabilized collagenase. Collagenase treated according to the process herein before lyophilization will reproducibly solubilize and maintain a fairly consistent level of activity over an extended period of time. In contrast, untreated collagenase will be severely degraded by the lyophilization process and

storage, with a corresponding loss of solubility in aqueous solutions. Further, the invention allows these beneficial properties to be realized using small amounts of inexpensive, and easily obtained materials without labor intensive procedures. Moreover, this process also has the advantage of yielding a product which acts predictably over extended periods of time.

Those skilled in the art will appreciate that the concentration and activity of collagenase present in the initial solution will vary with the starting material and enzyme purification procedure, if any. The well known principles of enzyme activity are applicable. Basic experimentation involving techniques designed to optimize and quantify enzyme concentration and total activity can provide the necessary information to determine the initial parameters. Common protein quantification methodology such as enzyme activity assays, electrophoresis, chromatography or spectroscopy may be used for the determination of overall enzyme concentration. The stability of the collagenase may be determined from the specific activity of the enzyme obtained through the use of collagenase assays well known in the art.

While dried purified forms of collagenase are available from a number of commercial sources including Sigma Chemical Company of St. Louis, MO., and Boehringer Mannheim Biochemicals of Indianapolis, IN., they are sometimes very difficult to dissolve due to denaturation.

Collagenase may be stored as an aqueous buffered solution. Those skilled in the art will recognize there are a number of ways to alter the composition of the buffers and concentration of the enzyme to manipulate storage parameters. In a preferred embodiment of the invention, the collagenase buffer is altered through extensive dialysis of the enzyme. Similarly, the concentration of the enzyme may be adjusted, for example, through ultrafiltration, to preferred levels which are optimized to increase the efficiency of the process. While the levels of enzyme in the starting material may be altered to correspond to production considerations, typical collagenase concentrations in the purified starting material are on the order of about 0.5 mg/ml to about 25 mg/ml.

As previously indicated, the collagenase is generally concentrated and introduced into a buffer solution in preparation for mixing with the selected stabilizer. In a preferred embodiment of the present invention, the stabilizer is dissolved in the same aqueous buffer solution as the collagenase. However, there is no requirement that the collagenase buffer and stabilizer buffer be identical. The buffers may vary in both their physical properties and volumes added to the mixing vessel. Those familiar with the art will recognize that there are a number of physiologically compatible buffers which can provide a suitabl medium for the desired

components. Where phosphate buffered saline (PBS) is used it can be used in a range of about pH 5 to pH 8.5. In one embodiment of the invention, both the buffer containing the stabilizer and the buffer present in the initial collagenase solution are a 0.1 M PBS, pH 7.6, having small amounts of other salts present. Further, the buffer may contain a nonionic surfactant such as Tween-20, available from SIGMA Chemical Co., present in low concentrations.

In a preferred method of practicing the invention a predetermined amount of collagenase in the desired buffer is combined with a corresponding amount of stabilizing compound or compounds, also in a selected buffer. Additional buffer may then be added to bring the combination up to its final mixing volume. As the final mixing volume will be greater than either the volume of the stabilizing solution or the collagenase solution, allowances must generally be made for the dilution of the enzyme or stabilizer. However, in other embodiments of the process, the concentrated collagenase solution may simply be diluted with buffer to obtain the final mixing volume before a solid form of the stabilizer is added. The final mixing volume, which will eventually be lyophilized, may be adjusted according to material limitations and production needs.

The preferred stabilizing agents used for the invention, as described above, consist of selected oligosaccharides or globulins. In an esp cially preferred

embodiment the globulin is human serum albumin (HSA), which may be obtained from a number of sources. HSA from Baxter-IV Systems is especially suitable because it has a proven long shelf-life in solution and is approved for in vivo use. Preferably, the oligosaccharide is either trehalose, maltose, or sucrose. However, any oligosaccharide with similar properties can be used and will give similar results. Properties indicative of compatibility may include, but are not limited to, hydrophilic and hydrogen bonding properties, solubilization parameter, molecular weight, molecular charge, and isoelectric point.

The final concentration of stabilizer sufficient to maintain enzymatic activity is dependent on the physical parameters used in practicing the invention as well as the particular stabilizer selected. Generally favorable stabilization effects have been obtained with stabilizer concentrations of about 0.05% to about 20% of the final mixing volume under compatible conditions. Especially preferred are concentrations of about 0.5%.

The lyophilization volumes are determined by the type of apparatus used and optimized according to production demands. For example, a typical lyophilizer is one manufactured by VirTis, which is used with starting aliquot volumes of 5 mL per vial. The lyophilization is continued until the sample is completely desiccated to a dry powder. The vials may be capped, sealed, and stored,

or the material may be collected and packaged according to its intended use.

In order to assess the post-lyophilization recovery and any degradation of the collagenase during storage, enzymatic activity assays are performed. The assay performed can be essentially the same whether the parameters are designed to test the lyophilization recovery or to document the effects of stabilizers on the retention of hydrolytic activity over a long period of time.

The collagenase activity is assayed by using a FALGPA, furylacryloyl Leu-Gly-Pro-Ala, synthetic substrate. The activity may be reported in nkat/ml and, when taken as a percentage of a control baseline, may be used as a convenient gauge to determine the retention of hydrolytic activity following lyophilization and/or storage. The nkat/ml unit is defined as nanomoles of substrate hydrolyzed per second by 1.0 ml of enzyme solution under the assay conditions used.

As a control, aliquots of the final reaction volume containing stabilizer and collagenase are frozen at -80°C and thawed at the appropriate time. The lyophilized samples, whether stored or fresh, are rehydrated with the appropriate volume of deionized H_2O. The volumes are adjusted to provide equivalent concentrations of collagenase in the control and lyophilized samples. The reconstituted collagenase is then assayed to determine the amount of activity remaining.

In the following examples purified collagenase was used because the compositions of the solutions can be better defined than in the case of crude collagenase.

The following example illustrates the effect of stabilization of collagenase during lyophilization.

Example 1

Collagenase was purified according to the method described in Bond et al., "Purification and Separation of Individual Collagenases of Clostridium histolyticum Using Red Dye Ligand Chromatography", Biochemistry Vol. 23, No. 13, 3077-3091, 1984. Concentrated solutions of 10% trehalose, 10% sucrose, or 10% HSA stabilizer were mixed in PBS buffer and filtered to remove impurities. A quantity of 0.5 mL of collagenase solution was combined with 5 mL of stabilizer solution or

10 μL of 10% TWEEN 20 solution. The combined solutions were then adjusted to 10 mL with PBS and stirred slowly for a period of 5 min. at 4°C.

The resulting stabilized collagenase solutions were adjusted to a concentration of approximately 18μg/ml in the final mixing volume of each solution. The individual stabilizer present in each solution had a concentration of 5% of the final mixing volume, except TWEEN 20, which had a concentration of 0.01%. Following stirring, samples were immediately frozen at -80°C or frozen and then lyophilized. Assays were perf rmed after the samples were lyophilized and rec nstituted with DI water to their

original v lume. The frozen (-80°C) samples were thawed and assayed as 100% control. Percent activity recovered was determined by taking the activity of the reconstituted lyophilized sample in nkat/ml and dividing that by the activity of the liquid control. The results are shown in Table I.

TABLE I

Retained Collagenase Activity Following Lyophilization

Additives	Control	TWEEN 20 0.01%	Sucrose 5%	Trehalose 5%	HSA 5%
Liquid	82.5	100.7	88.2	90.8	83.4
Lyophilized	34.0	37.8	73.6	91.6	80.1
% Recovered	41	38	83	101	96

From the data in Table I it is obvious that the addition of a stabilizer according to the present invention results in a dramatic increase in activity retention. Both the addition of 5% trehalose and 5% human serum albumin resulted in almost no hydrolytic activity being lost. In contrast, the buffered control and the buffered control with 0 .01% Tween-20 lost well over half their activity over the same time period.

The following example illustrates the retention of hydrolytic activity over a long period of time under

different c nditions. In this case only one stabilizing agent was used so as to reduce the variation in the study and demonstrate the effects of different temperatures and buffers on stabilized collagenase over time.

Example 2

The procedure of Example 1 was followed except that human serum albumin was used as the stabilizer and TWEEN-20 was not used. Vials of lyophilized samples were stored at 4°C, room temperature, or 37°C during the course of the experiment. At periodic intervals, lyophilized samples were reconstituted with water and assayed along with a frozen control aliquot to determine remaining hydrolytic activity. The results are shown in Table II.

TABLE II

Collagenase Activity Retained Over An Extended Period Without TWEEN-20

	Days stored	Liquid -80°C	Lyophilized 4°C	Lyophilized Room Temp.	Lyophilized 37°C
10	0	77.94	71.53	71.53	71.53
	3	88.60	76.54	73.65	75.70
	7	82.27	75.47	77.14	73.08
	10	89.43	77.94	76.84	77.25
	21	82.93	75.43	74.95	74.96
15	28	82.00	75.34	76.24	76.89
	35	85.11	80.61	80.84	79.46
	49	82.74	80.08	79.25	80.49
	63	82.62	80.14	80.27	71.57

As the results indicate very little activity was lost during the 63 days of the experiment, and clearly there is no trend in activity change. Furthermore, there does not appear to be a statistical difference in the amount of activity retained by the samples stored at different temperatures. The small difference in activities may be due to variance in reconstitution of samples. Such unexpect d results provide strong evidence that the

stabilization of collagenase with human serum albumin will result in consistent enzymatic activity over a long period of time under a myriad of storage conditions. By way of comparison non-stabilized commercial collagenase loses its activity upon extended storage.

The following example illustrates the effect of using a surfactant along with the stabilizer.

Example 3

The procedure of Example 2 was followed except that 0.01% TWEEN-20 was used with the buffer. The results are shown in Table 3.

TABLE III

Collagenase Activity Retained Over An Extended Period With 0.01% TWEEN-20

Days Stored	Liquid -80°C	Lyophilized 4°C	Lyophilized Room Temp.	Lyophilized 37°C
0	78.87	70.25	70.25	70.25
3	88.43	76.01	75.32	74.83
7	89.79	76.79	74.43	74.21
10	97.52	77.07	74.64	76.73
21	82.46	77.35	76.57	74.90
28	81.56	78.13	77.25	73.99

35	86.04	80.17	79.90	79.31
49	82.82	79.96	81.64	79.59
63	82.38	79.13	78.62	76.23

The results show that very little activity was lost whether or not a surfactant was incorporated with the buffer.

Having thus described preferred exemplary embodiments of the present invention, it should be noted by those skilled in the art that the disclosures herein are exemplary only and that alternatives, adaptions and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments illustrated herein.

Claims:

1. A solid stabilized collagenase composition comprising the product of lyophilization of a mixture comprising collagenase and an effective amount of a collagenase stabilizer.

2. A composition according to claim 1 wherein said stabilizer is a member selected from the group consisting of globulins and non-reducing sugars.

3. A composition according to claim 1 wherein said stabilizer is an oligosaccharide.

4. A composition according to claim 3 wherein said oligosaccharide is a non-reducing sugar selected from the group consisting of trehalose, sucrose, and maltose.

5. A composition according to claim 1 wherein said stabilizer is a globulin selected from the group consisting of human serum albumin and bovine serum albumin.

6. A process for producing a solid stabilized collagenase composition which comprises:

solubilizing crude collagenase in an aqueous solution;

combining the solubilized collagenase with an effective amount of a stabilizer and a buffer solution having a pH ranging from 5.0 to 8.5 to generate a stabilized collagenase solution; and

lyophilizing said stabilized collagenase solution to produce a solid stabilized collagenase composition.

7. The process of claim 6 which additionally comprises the step of purifying the solubilized crude collagenase.

8. A process according to claim 6 wherein said stabilizer comprises a member selected from the group consisting of globulins and non-reducing sugars.

9. A process according to claim 6 wherein said stabilizer is an oligosaccharide.

10. A process according to claim 8 wherein said oligosaccharide is a non-reducing sugar selected from the group consisting of sucrose, trehalose, and maltose.

11. A process according to claim 6 wherein said stabilizer is a globulin selected from the group consisting of human serum albumin and bovine serum albumin.

12. A process according to claim 6 wherein the concentration of stabilizer in said stabilized collagenase solution is about 1 mg/ml to about 100 mg/ml.

13. A process according to claim 6 wherein said buffer solution is phosphate buffered saline having a pH of about 5.0 to about 8.5.

14. A process according to claim 6 wherein said buffer solution comprises a nonionic surfactant in a concentration of about 0.01% to about 0.05%.

15. A process according to claim 6 which additionally comprises the step of adding additional buffer solution to said stabilized collagenase solution to provide a convenient final mixing volume.

16. A process according to claim 15 wherein said stabilizer is present in a concentration of about 0.05% to about 20% of said final mixing volume.

17. A solid stabilized c llagenase composition produced according to the process of claim 6.

18. A solid stabilized collagenase composition produced according to the process of claim 7.

19. A method for dissociating connective tissue which comprises digesting said tissue with an effective amount of the composition of claim 1.

20. A method according to claim 14 wherein said connective tissue is associated with <u>in vitro</u> tissue culture cells.

21. A method for clinically treating a burn or an ulcer which comprises contacting said burn or ulcer with the composition of claim 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/04082

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N9/96 C12N9/52 A61K37/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 260 645 (KNOLL AG) 23 March 1988 see the whole document ---	1,2,5-8, 11-18,21
X	EP,A,0 468 180 (SCLAVO S. P. A.) 29 January 1992 see the whole document ---	1,2,17, 18,21
X	EP,A,0 115 974 (INSTITUT PASTEUR) 15 August 1984 cited in the application see page 19 - page 20 --- -/--	1,17,18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1994	- 4. 08. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	**Authorized officer** Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/04082

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 9219, Derwent Publications Ltd., London, GB; Class B04, AN 92-156055 & JP,A,4 094 685 (MORISHITA JINTAN KK) 26 March 1992 see abstract -----	1-4, 6-10, 12-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04082

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claim 21 is directed to a method of treatment of (diagnostic method practised on) the human/animal body (Article 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International application No.
PCT/US 94/04082

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